Bile acid promotes intestinal metaplasia and gastric carcinogenesis

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Running Title; Bile acids regulate gastritis and promote cancer

Key words: bile acid, gastritis, CDX2, CINC1, Helicobacter pylori

Abbreviations: H. pylori, Helicobacter pylori; PG, pepsinogen; CA, cholic acid; CDC, chenodeoxycholic acid; GCA, glycocholic acid; GCDC, glycochenodeoxycholic acid

Financial support; none

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Conflict of interest; none

The word count: 2,462 The total number of figures: 5
ABSTRACT

Background: Bile acid and *Helicobacter pylori* are important toxic factors for gastric mucosal injury. We examined the role of bile acid in promoting histological gastritis and gastric carcinoma in Japanese patients.

Methods: A total of 767 patients (452 men, mean age 51.1 y.o.) were studied. Gastric juice was collected by gastro-endoscopic examination, and the bile acid concentration was examined by enzymatic method. The grade of histological gastritis was evaluated by gastric biopsies, and the relationship between the bile acid concentration and the gastritis score was examined. The occurrence of gastric cancer was examined by a retrospective cohort study. *CDX2/CINC1* expression in RGM-1 cells was evaluated by real-time PCR.

Results: In *H. pylori*-positive patients, we found significant positive correlation between the bile acid concentration and the grades of atrophy/intestinal metaplasia (*P*<0.01). However, we found significant negative associations between the bile acid concentrations and the histological scores of mononuclear cell/neutrophil infiltrations (*P*<0.01). Patients with a high concentration of bile acid developed gastric cancer more frequently than those with a low concentration (*P*<0.05). Cholic acid treatment significantly increased *CDX2* expression in RGM-1 cells. *CINC1* expression in RGM-1 cell was significantly induced by co-culture with *H. pylori* and the induction was reduced by glycochenodeoxycholic acid treatment.

Conclusion: The bile acid in gastric juice contributes to the progression of histological atrophy and intestinal metaplasia without inflammatory cell infiltration, followed by carcinogenesis in *H. pylori*-positive patients.

Impact: Bile acid promotes intestinal metaplasia and gastric carcinogenesis without inflammatory cell infiltration.
INTRODUCTION

*H. pylori* plays an important role in the induction of chronic gastritis, and there is a strong association between *Helicobacter pylori* (*H. pylori*)-associated gastritis and gastric diseases including peptic ulcer, functional dyspepsia, and gastric cancer (1). The basic components of the process are chronic active non-atrophic gastritis → multifocal atrophy → intestinal metaplasia → dysplasia → invasive carcinoma (2).

However, in *H. pylori*-positive patients, the progression of atrophic gastritis is quite different among patients with infection. It is still unclear whether some patients showed advanced progression of gastritis followed by gastric carcinogenesis. Many studies have revealed that multiple factors including bacterial factors, environmental factors, and the host immune response contribute to the progression of mucosal atrophy, metaplasia, and dysplasia toward gastric cancer (3;4).

Bile acid is another important toxic factor involved in the injury of gastric mucosa (5). In the remnant stomach of rats after gastrectomy, bile acid, the main component of the duodenal juice, has been implicated in gastric cancer due to duodenogastric reflux (6). In humans, duodenogastric reflux also appears to be implicated in gastric stump carcinoma (7). Previous studies have demonstrated that bile acid was associated with intestinal metaplasia in the cardia (8) and with the degree of esophageal mucosal injury (9). Several clinical studies have emphasized the role of chronic duodenogastro-esophageal reflux in the development of Barrett’s esophagus with intestinal metaplasia (10;11). We previously reported that the bile acid caused DNA damage, which may contribute to the carcinogenesis (12).

The *CDX1* and *CDX2* homeobox transcription factors show intestine-specific expression in adult tissues and appear to have critical functions in intestinal development as well as in specification and maintenance of the intestinal phenotype in adults (13). The mouse *CDX1* and *CDX2* genes are expressed rather broadly in caudal structures during the early stages of embryonic development, but in the later stages of development and in normal adult tissues, expression of these genes are restricted to the epithelium of the small intestine and colon (14;15). Muto *et al.* directly demonstrated that *CDX2* contributes to the generation of intestinal metaplasia of gastric epithelial cells in transgenic mice (16). Cytokine-induced neutrophil chemoattractant-1 (*CINC1*), which corresponds to human interleukin-8 (IL-8), is a key cytokine in the rat. Previous studies demonstrated that CINC1 protein plays an important role in rat gastric inflammation induced by *H. pylori* infection (17).

In the present study, we tried to clarify the role of the bile acid in gastric juice in gastric inflammation/carcinogenesis. We examined (1) the relationship between the
bile acid concentration in gastric juice and histological gastritis in a large-scale cross-sectional study, (2) the relationship between the bile acid concentration in gastric juice and gastric cancer occurrence in Japanese patients with *H. pylori* in a retrospective cohort study. Furthermore, we investigated whether bile acid influences intestine-specific gene expression (*CDX2*) and inflammation-related cytokine (*CINC1*) in rat gastric epithelial cells cultured *in vitro*.
MATERIALS AND METHODS

Patients
Seven hundred and sixty-seven patients with dyspepsia (452 men, mean age 51.1 y.o.) were included in this study. We excluded patients with previous gastrectomy or with previous H. pylori eradication therapy. No patients who were continuously taking proton pump inhibitors were included in the study. All patients received routine gastroduodenal endoscopic examination between October 1996 and July 2006. We confirmed that no patient had gastric neoplasm, and the endoscopic features were recorded in a database. Gastric juice was collected by aspiration before routine observation. It was stored at -20 °C until use, and then pH and the concentration of bile acid were evaluated with the use of an enzymatic method (18). Type of bile acid was evaluated by gas chromatography. H. pylori infection was examined by histological examination and antibody titer (E-plate, Eiken, Japan). H. pylori was regarded as negative when two tests both showed negative results. A total of 357 patients (men 223, mean age 60.0 y.o.) out of 767 patients received endoscopic examination for cancer screening for less than 3 years (range 3-30 years, mean 10 years) and these data were recorded in the database in Hiroshima University Hospital. Ethics Committee of Hiroshima University Hospital approved our protocol (Hiroshima University Hospital #222).

Histological gastritis score
In upper gastrointestinal endoscopy, gastric biopsies (2 from the antrum and 2 from the corpus) were obtained. Biopsy specimens were taken from a site without localized lesions. Specimens were fixed with formalin and stained with hematoxylin and eosin. Gastritis scores were evaluated by two specialists (MI and MT) independently, using the updated Sydney system (19).

Cell culture and RNA extraction
Rat gastric epithelial cell line (RGM-1), which was authenticated by RIKEN BioResource Center (Tsukuba, Japan), was purchased from RIKEN cell bank (Tsukuba, Japan). These cells were routinely cultured in a 1:1 mixture of Dulbecco’s modified Eagle’s medium (DMEM) and Ham’s F12 medium supplemented with 10% heat-inactivated fetal bovine serum. In the examination of CDX2/CINC1 expression, four kinds of bile acid (200 μM cholic acid (CA), chenodeoxycholic acid (CDC), glycocholic acid (GCA), or glycochenodeoxycholic acid (GCDC)) with H. pylori were added to the culture medium for 24 hours before harvesting. H. pylori strain was
derived from a patient with gastric ulcer and we confirmed that it was CagA-positive (East Asian type) (20). After incubation in the liquid culture medium for 24 hours, *H. pylori* was added to the culture supernatant of RGM-1 cells.

**RNA extraction and PCR reaction**

RNA was extracted from RGM-1 cells with an RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. Reverse transcription polymerase chain reaction (RT-PCR) was performed using a first-strand cDNA kit (GE Healthcare UK Ltd., Little Chalfont, England). One-microliter aliquots of the synthesized cDNA were mixed with SYBR Green PCR master mix (Applied Biosystems, Foster City, CA) with appropriate primers and amplified using a real-time PCR system 7300 (Applied Biosystems, Foster, CA, USA). Sense and antisense primers used were 5’-aga aca tcc aga gtt tga agg tga t-3’ and 5’-gtg gct atg act tcg gtt tgg-3’ (21) for *CINC1* (67 bp), 5’-tgt gtc gta aat gcc aga gc-3’ and 5’-acc ctc ata gat ggg cac ag-3’ (NCBI database NM-023963) for *CDX2* (77 bp), and 5’-cag caa ttc cgg tct tct tc-3’ and 5’-acc ctc ata gat ggg cac ag-3’ (22) for GAPDH (288 bp), respectively. The specificity of each amplified product was confirmed by dissociation analyses giving a single sharp dissociation peak, the absence of the amplified product without reverse transcription, and the appearance of a band of the expected size on electrophoresis of the amplified product.

The expression of *GAPDH* was used as an internal control. All quantified values were normalized to those of *GAPDH* (quantified value for a certain target/quantified value for *GAPDH*).

**Statistical analysis**

Statistical analysis was performed using the Mann-Whitney U-test, Kaplan-Meier method, Kruskal-Wallis test, and Steel test with Stat View (SAS Institute Inc., Cary, NC). A value of *P*<0.05 was considered significant.
RESULTS

Relationship between bile acid concentration and histological gastritis

Out of 767 patients examined, 612 were regarded as H. pylori-positive. First, we examined the relationship between histological gastritis and bile acid concentration in the gastric juice in H. pylori-positive patients. As demonstrated in Figure 1, bile acid concentration was significantly higher in the group with high atrophy/intestinal metaplasia and with low activity/inflammation in the gastric antrum. The same result was obtained for the gastric corpus (data not shown). However, in H. pylori-negative patients, the bile acid concentration was significantly low and such a tendency was not detected either in the gastric antrum or in the corpus (Figure 2). Next, we excluded the patients with marked atrophy, and carried out the same examination to refute the influence of natural clearance of H. pylori followed by decreased cell infiltration. Surprisingly, the bile acid concentration was significantly lower in the group with a high score for gastric inflammation (Figure 3).

Bile acid concentration and gastric carcinogenesis

Next we conducted a retrospective cohort study to investigate the role of bile acid in gastric carcinogenesis. Retrospectively, we enrolled 357 patients who were followed by endoscopic examination for cancer screening for less than 3 years, and conducted a cohort study using these patients. We set the cut-off at 1,000 μM and grouped the patients by high and low concentrations of bile acid. As illustrated in Figure 4, on the basis of the Kaplan-Meier method, we found that the prevalence of gastric cancer development was statistically higher in patients with high concentrations of bile acid. Gastric cancers that developed in these patients were 28 intestinal-type and 7 diffuse-type cancers.

Effect of bile acid on the expression of CDX2 and CINC1 in RGM-1 cells

We next examined the effect of bile acid on gastric mucosa cells, which were treated with 200 μM cholic acid (CA), chenodeoxycholic acid (CDC), glycocholic acid (GCA), and glycochenodeoxycholic acid (GCDC), and then we examined the CDX2/CINC1 expression in gastric epithelial cells in vitro. CDX2 expression level was not altered by co-culture with H. pylori. However, CDX2 level was statistically higher in the group that underwent CA treatment than in the control (P<0.05, Figure 5A). This effect was not clear for other kinds of bile acids on RGM-1. On the other hand, CINC1 expression level was extremely low in RGM-1 cells at the basal status; however, its expression was significantly induced by co-culture with H. pylori (Figure 5B).
GCDC was added, *CINCl* expression was significantly suppressed (P<0.05). In contrast, unconjugated bile acids (CA and CDC) increased *CINCl* expression. The effect on *CINCl* expression was significantly different between glycine-conjugated and unconjugated bile acids.
DISCUSSION

In the present study, we demonstrated that in *H. pylori*-positive patients (1) a significant correlation was found between the bile acid concentration and atrophy/IM, (2) a significant negative association was disclosed between bile acid concentration and inflammatory score as histologically assessed in gastric mucosa biopsy samples. In addition, we found that (3) patients with a high concentration of bile acid developed gastric cancer more frequently than those with a low bile acid concentration. These suggest that bile acid in gastric juice contributes to the progression of histological atrophy and intestinal metaplasia without inflammatory cell infiltration, followed by carcinogenesis, in *H. pylori*-positive patients.

In addition, we tried to clarify the mechanism of these alterations using RGM-1 cells in vitro. We confirmed CDX2 induction in RGM-1 cells after CA treatment. Xu et al. recently reported that CDC induced CDX2 expression in RGM-1 cells (23). Recent studies focused on the development of intestinal metaplasia on esophago-gastric junction leading to Barrett esophagus (24). Our previous work demonstrated that bile acid has an effect in promoting gastric atrophy and intestinal metaplasia (25).

However, the inhibition of inflammatory cell infiltration was quite unexpected. As far we know, this is the first report describing the inhibitory effect of bile acid on gastric inflammation induced by *H. pylori* infection. It is hypothesized that decreased inflammation may arise from severe atrophy and natural clearance of *H. pylori*. In addition, it is well known that bile acid has an anti-*H. pylori* effect (26). However, surprisingly, our additional result (Figure 3) contradicts this hypothesis because we could also show a negative association between bile acid inflammation and grades of mononuclear cell infiltration, even in patients with no or mild atrophy, in whom *H. pylori* was not cleared. These results support the anti-inflammatory effect of bile acid in the gastric juice.

In the present study, we demonstrated that CINCI, which encodes the rat cytokine similar to human interleukin (IL)-8 (27), was dramatically induced by *H. pylori* infection and was inhibited by GDGC treatment in RGM-1 cells. As is well known, IL-8 is a key cytokine that enhances *H. pylori*-induced gastric inflammation showing mononuclear cell/neutrophil infiltration in the mucosal layer (28). We demonstrated the possibility that bile acid firstly has an inhibitory effect on CINCI expression induced by *H. pylori* infection, and secondly inhibits the infiltration of inflammatory cells in the gastric mucosa. Of course, the anti-bacterial effect of bile acid may play a partial role in inhibiting the infiltration of inflammatory cells.

It is questionable whether intestinal metaplasia without inflammatory cell infiltration...
can be linked to gastric carcinogenesis. The intestinal metaplasia induced by bile acid was not a result of a secondary event following active inflammation, but rather a direct effect of bile acid. Therefore, we conducted a retrospective cohort study in our subjects to analyze gastric cancer development. Interestingly, we could demonstrate a positive relationship between bile acid concentration and gastric cancer development, suggesting the carcinogenic role of bile acid-induced gastritis or intestinal metaplasia. Since this mucosa has few inflammatory cells, the indirect effect from inflammatory cells seems to be unlikely by, for example, nitric oxide release by inflammatory cells (29). A recent study demonstrated that bile acid directly affects intracellular signaling of gastric epithelial cells (30). Besides CDX2, the alteration of carcinogenic pathways should be clarified in future studies.

As demonstrated in our results, the proportion of different fractions in bile acid is quite important. From our previous results, the major components of bile acids were CA and CDC (31); however, it was shown that the greater part in gastric juice was glycine-conjugated type (GCA and GCDC) (32). Therefore, we used these four bile acids in the present study. It is well known that bile acids do not equally regulate gastric inflammation or carcinogenesis (33). For example, we confirmed that CA selectively increased CDX2 expression in vitro. On the other hand, the decrease of CIN1 expression was clear in glycine-conjugated bile acids, especially in GCDC. Concerning the CIN1-related inhibition of inflammatory cell infiltration in particular, the process of glycine conjugation may be important. In addition, we previously demonstrated the specific carcinogenic role of GCDC, suggesting the exceptional role of GCDC in gastric inflammation and carcinogenesis (12). It will be important to evaluate bile acid proportions and clinical outcome in future studies. Since our study design was a cross-sectional retrospective cohort, a prospective cohort study is needed to clarify the anti-inflammatory but carcinogenic effect of bile acid.

In the present study, the bile acid titer was significantly lower in H. pylori-negative patients. The reason for this difference is unknown. We previously demonstrated that the degree of duodenal-gastric reflux was statistical higher in H. pylori-positive patients than in -negative patients by ultrasonographic examination (34). However, this difference was not so pronounced. While this may be a partial explanation, the main reason is still unclear. The difference in gastric juice pH may be important (35).

In Japan, the prevalence of H. pylori infection is decreasing gradually and the spread of eradication therapy has accelerated the disappearance of this bacterium. We will be able to control the carcinogenic effect of H. pylori in the near future (36;37). Although H. pylori-negative gastric cancer is rare (38), the role of bile acids should become
important in gastric carcinogenesis in the next generation. Therefore, we should clarify the detailed mechanism by which bile acid induces gastric inflammation and carcinogenesis.
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Figure Legends

Figure 1. Relationship between bile acid concentration and gastritis score (atrophy (A), intestinal metaplasia (B), inflammation (C), activity (D)) in the gastric antrum of *H. pylori*-positive patients. The titer of bile acid concentration is illustrated by a linear scale. *, P<0.05

Figure 2. Relationship between bile acid concentration and gastritis score (atrophy (A), intestinal metaplasia (B), inflammation (C), activity (D)) in the gastric antrum of *H. pylori*-negative patients. The titer of bile acid concentration is illustrated by a linear scale. *, P<0.05

Figure 3. Relationship between bile acid concentration and inflammation score in the corpus in patients with no to moderate atrophy. The titer of bile acid concentration is illustrated by a linear scale. *, P<0.05

Figure 4. Kaplan-Meier analysis of gastric cancer development. Patients were sub-classified into two groups: high bile acid (≥1,000 μM) and low bile acid (<1,000 μM) groups.

Figure 5. The expression of CDX2 and CINC1 in RGM-1 cells. Cells were incubated with or without 200 μM bile acid/*H. pylori* for 24 hours, and then real-time PCR was performed. (A) Expression level of CDX-2 evaluated by real-time PCR (n=4). (B) Expression level of CINC1 evaluated by real-time PCR (n=5). The relative expression level (CDX2/GAPDH or CINC1/GAPDH) was expressed by setting the standard (*H. pylori* positive and no bile acid) as 1.0. Bars, SEM. *P<0.05
Tatsugami et al. Figure 4

Gastric cancer-free survival rate

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Cancer Epidemiol Biomarkers Prev  Published OnlineFirst September 25, 2012.

Updated version

Access the most recent version of this article at:
doi:10.1158/1055-9965.EPI-12-0730

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